



TITLE:

Production of pyruvate from mannitol by mannitol-assimilating pyruvate decarboxylase-negative *Saccharomyces cerevisiae*.

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1 **Production of pyruvate from mannitol by mannitol-assimilating pyruvate**
2 **decarboxylase-negative *Saccharomyces cerevisiae***

3

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15 Running Head: Production of pyruvate from mannitol

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18 **Abstract**

19 Mannitol is contained in brown macroalgae up to 33% (w/w, dry weight), and
20 thus is a promising carbon source for white biotechnology. However,
21 *Saccharomyces cerevisiae*, a key cell factory, is generally regarded to be unable to
22 assimilate mannitol for growth. We have recently succeeded in producing *S.*
23 *cerevisiae* that can assimilate mannitol through spontaneous mutations of
24 Tup1-Cyc8, each of which constitutes a general corepressor complex. In this study,
25 we demonstrate production of pyruvate from mannitol using this
26 mannitol-assimilating *S. cerevisiae* through deletions of all three pyruvate
27 decarboxylase genes. The resultant mannitol-assimilating pyruvate
28 decarboxylase-negative strain produced 0.86 g/L pyruvate without use of acetate
29 after cultivation for 4 days, with an overall yield of 0.77 g of pyruvate per g of
30 mannitol (the theoretical yield was 79%). Although acetate was not needed for
31 growth of this strain in mannitol-containing medium, addition of acetate had a
32 significant beneficial effect on production of pyruvate. This is the first report of
33 production of a valuable compound (other than ethanol) from mannitol using *S.*
34 *cerevisiae*, and is an initial platform from which the productivity of pyruvate from
35 mannitol can be improved.

36

37 Key words: pyruvate, mannitol, brown macroalgae, pyruvate decarboxylase,
38 *Saccharomyces cerevisiae*

39 Introduction

40 Mannitol is a sugar alcohol derivative of mannose and a promising carbon
41 source for white biotechnology, since brown macroalgae contains mannitol at up to
42 33% (w/w, dry weight) ¹⁻³. The budding yeast *Saccharomyces cerevisiae* is a key
43 cell factory that is used for production of a wide range of industrial products ⁴.
44 However, *S. cerevisiae* including the S288C reference strain is generally thought to
45 be unable to assimilate mannitol for growth ⁵. However, we ⁶ and Enquist-Newman
46 *et al.* ⁷ have recently succeeded in producing *S. cerevisiae* that can utilize mannitol,
47 thus opening a new way to produce valuable compounds from mannitol.

48 Enquist-Newman *et al.* overexpressed genes for mannitol dehydrogenase and
49 mannitol transporter and produced a *S. cerevisiae* strain that assimilates mannitol ⁷.
50 We also produced a *S. cerevisiae* strain that assimilates mannitol using spontaneous
51 mutations of Tup1-Cyc8, each of which constitute a general corepressor complex
52 that regulates many genes ⁶. We demonstrated production of ethanol from mannitol
53 using this *S. cerevisiae* strain ⁶. These mannitol-assimilating *S. cerevisiae* strains
54 may also have potential for production of other valuable compounds from mannitol.

55 Pyruvate is widely used for production of crop-protection agents, polymers,
56 cosmetics, and food additives, and as a starting material in the biosynthesis of
57 pharmaceuticals (e.g., L-DOPA, alanine, L-tryptophan, and L-tyrosine) ^{8, 9}.
58 Pyruvate production from glucose has been achieved using a pyruvate
59 decarboxylase (Pdc)-negative *S. cerevisiae* TAM strain ¹⁰. The goal of this study
60 was to produce pyruvate from mannitol using our mannitol-assimilating *S.*
61 *cerevisiae* strain ⁶.

62

63 **Materials and Methods**

64 Strains and Media

65 The *S. cerevisiae* strains used in the study are listed in Table 1. *ADE2* of
66 MK4416 was removed and *TRP1* was replaced with *trp1Δ63* using plasmid
67 YRp14-*trp1Δ63* (ATCC 77148)¹¹, resulting in the MK5316 strain. *PDC1* and
68 *PDC6* of MK5316 were eliminated¹¹ and *PDC5* was deleted¹², resulting in the
69 MK5376 strain. Detailed information is described in Supplementary Methods.
70 Standard yeast media were used¹³, including SG, SM, SGE (ethanol 0.15%), and
71 SGE (ethanol 0.3%) media consisting of 0.67% w/v yeast nitrogen base w/o amino
72 acids (BD), complete amino acids/nucleosides (Clontech), and carbon sources: 2%
73 v/v glycerol (SG), 2% w/v mannitol (SM), 2% v/v glycerol plus 0.15% v/v ethanol
74 [SGE (ethanol 0.15%)], and 2% v/v glycerol plus 0.3% v/v ethanol [SGE (ethanol
75 0.3%)]. Glycerol (30% v/v) and sodium acetate (27.1% w/v, pH 9.1; equal to 20%
76 w/v acetate) were each autoclaved separately from the other components and
77 ethanol was also separately sterilized by filtration (0.20-μm pore size). Liquid
78 medium was solidified at 2% w/v agar. Amino acids/nucleosides are removed from
79 the medium when necessary. 5-Fluoroorotic Acid (FOA) for the counter-selection
80 of yeast was added to the solid medium at 1 mg/ml (26). The MK5316 and
81 MK5376 strains were maintained on SGE (0.3% ethanol) solid medium at room
82 temperature and stored in the presence of 17% v/v glycerol at -80°C. Cultivation
83 was conducted with a Personal Lt-10F (Taitec, Tokyo, Japan).

84

85 Pyruvate Production from Mannitol

86 Pyruvate production was conducted as follows, unless otherwise stated. The

87 MK5376 strain was precultured on SM solid medium, transferred to SM liquid
88 medium (5 mL in a 100-mL Erlenmeyer flask), and cultured at 30°C at 145 strokes
89 per min (spm) for approximately 24 h. Cells precultured in each medium were
90 transferred to SM liquid medium (10 mL in a 100-mL Erlenmeyer flask) and
91 further cultured at 0, 95, or 145 spm at 30°C.

92

93 Analytical Methods

94 Cultures were centrifuged at 20,000 g for 5 min at 4°C and each component in
95 the supernatant was analyzed. Ethanol was assayed using an Ethanol Assay F-kit
96 (Roche). The concentration of mannitol was determined using an HPLC equipped
97 with an Aminex HPX-87H (300 × 7.8 mm) (Bio-Rad) column (65°C, elution with 5
98 mM H₂SO₄ at 0.65 ml/min) and a RID-10A detector (Shimadzu) ⁶. Pdc activity was
99 assayed as described elsewhere ¹⁴. The protein concentration was determined using
100 a Bradford reagent assay (Sigma) ¹⁵ with bovine serum albumin as a standard.

101

102 Results and Discussion

103 Growth Phenotype of Mannitol-assimilating Pdc-negative *S. cerevisiae*

104 We previously found that *S. cerevisiae* BY4742 cells capable of assimilating
105 mannitol arise spontaneously from wild-type BY4742 cells during prolonged
106 culture in mannitol-containing medium due to spontaneous mutations in genes
107 encoding Tup1 or Cyc8, which constitute a general corepressor complex ⁶. Of the
108 strains that acquired the ability to assimilate mannitol, the MK4416 strain had a
109 spontaneous partial deletion in *CYC8* (Table 1) and showed salt tolerance, as well
110 as high ethanol productivity ⁶, and was chosen as the mannitol-assimilating strain in

111 this study. Auxotrophy for Ade and Trp was introduced into the MK4416 strain to
112 give the MK5316 strain, which was used as the parental mannitol-assimilating
113 strain. The three genes for Pdc in *S. cerevisiae* (*PDC1*, *PDC5*, and *PDC6*)¹⁶ were
114 deleted in the MK5316 strain to give the Pdc-negative and mannitol-assimilating
115 MK5376 strain (Table 1), which was confirmed to have no Pdc activity
116 (Supplementary Results).

117 The *S. cerevisiae* Pdc-negative strain in a T2-3D or CEN.PK 113-7D
118 background cannot grow in the presence of glucose in a defined liquid or solid
119 medium, but can grow in a glucose-limited chemostat culture in the presence of C2
120 compounds (ethanol or acetate)^{10, 14}. This requirement for C2 compounds was
121 attributed to a deficiency of this Pdc-negative strain to synthesize cytosolic
122 acetyl-CoA^{17, 18}. On the defined medium, the Pdc-negative MK5376 strain
123 exhibited no growth in the presence of glucose as reported^{10, 14}, but showed growth
124 in the presence of glycerol, glycerol plus ethanol, pyruvate, and, in particular, with
125 mannitol alone (Supplementary Fig. S1). Cytosolic acetyl-CoA would be supplied
126 from functional mitochondria when the MK5376 strain assimilates mannitol of
127 which assimilation requires functional mitochondrial respiration⁶. The
128 Pdc-negative MK5376 strain in the BY4742 background showed poorer growth in a
129 complex medium than in a defined medium (Fig. S1), in contrast to previous
130 findings for the Pdc-negative strain in a T2-3D background¹⁷.

131

132 Pyruvate Production from Mannitol

133 In a previous attempt to produce pyruvate from glucose using *S. cerevisiae*, a
134 Pdc-negative strain in a CEN.PK113-7D background was evolved to a TAM strain

[16]. The TAM strain was independent of C2 compounds and tolerant to glucose, and produced 135 g/L pyruvate with an overall yield of 0.54 g of pyruvate per g of glucose¹⁰. To produce pyruvate from mannitol using our MK5376 strain, the conditions for preculture of MK5376 strain were first examined. SG and SM media gave better pyruvate production than SGE (0.15 or 0.3% ethanol) media, although growth was not affected by these four media (Fig. 1A). Thus, we chose SM for preculture. Among the tested shaking speeds of 0, 95, and 145 spm, a speed of 0 spm (i.e., a static batch culture) resulted in the best production of pyruvate from mannitol (Fig. 1B). Using these conditions (preculture in SM medium and pyruvate production using a static batch culture), the pyruvate and ethanol productivity of the mannitol-assimilating Pdc-negative MK5376 strain was compared with that of the parental Pdc-positive MK5316 strain (Fig. 2). The parental strain produced no pyruvate, but the Pdc-negative strain produced 0.86 g/L pyruvate through consumption of 1.12 g/L mannitol after cultivation for 4 days, with an overall yield of 0.77 g of pyruvate per g of mannitol (the theoretical yield was 79%). This was a higher yield, but lower productivity, compared to the TAM strain (yield of 0.54 g of pyruvate per g of glucose, 135 g/L pyruvate production, consumption of 250 g/L glucose for 4 days)¹⁰. The difference in pyruvate productivity between the Pdc-negative strain MK5376 and the TAM strain could be attributed to fact that MK5376 metabolized mannitol less efficiently than TAM metabolized glucose, as indicated shown by the amount of sugar consumption (1.12 g/L mannitol vs 250 g/L glucose) and biomass formation (A_{600} of 1.7 [MK5376] vs. A_{600} of 50 [TAM strain]) after 4 days of cultivation (Fig. 2)⁴. The Pdc-negative strain MK5316 must acquire the enhanced ability to metabolize mannitol, e.g., through adaptive

159 evolution, as in the case of TAM.

160

161 Effect of Acetate on Pyruvate Production

162 The mannitol-assimilating Pdc-negative MK5376 strain showed no requirement
163 for C2 compounds, but there is a possibility that C2 compounds helped with supply
164 of cytosolic acetyl-CoA and enhanced pyruvate production. As expected, addition
165 of acetate up to 0.3% w/v had a significant effect on pyruvate production. First, the
166 strain produced pyruvate at shaking speeds of 95 and 145 spm (Fig. 1C), whereas
167 no pyruvate was produced at 95 and 145 spm in the absence of C2 compounds (Fig.
168 1B). Second, the strain produced a larger amount of pyruvate in the presence of
169 acetate compared to that in the absence of acetate (Fig. 1BC). After long-term
170 cultivation (20 days) at 0 spm, 2.71 g/L pyruvate was produced through
171 consumption of 2.2 g/L mannitol and 0.88 g/L acetate (Fig. S2). The mechanism
172 underlying this beneficial effect of acetate is unclear and further challenges are
173 needed to improve productivity of pyruvate. However, this is the first
174 demonstration of production of a valuable compound, other than ethanol, from
175 mannitol using *S. cerevisiae*.

176

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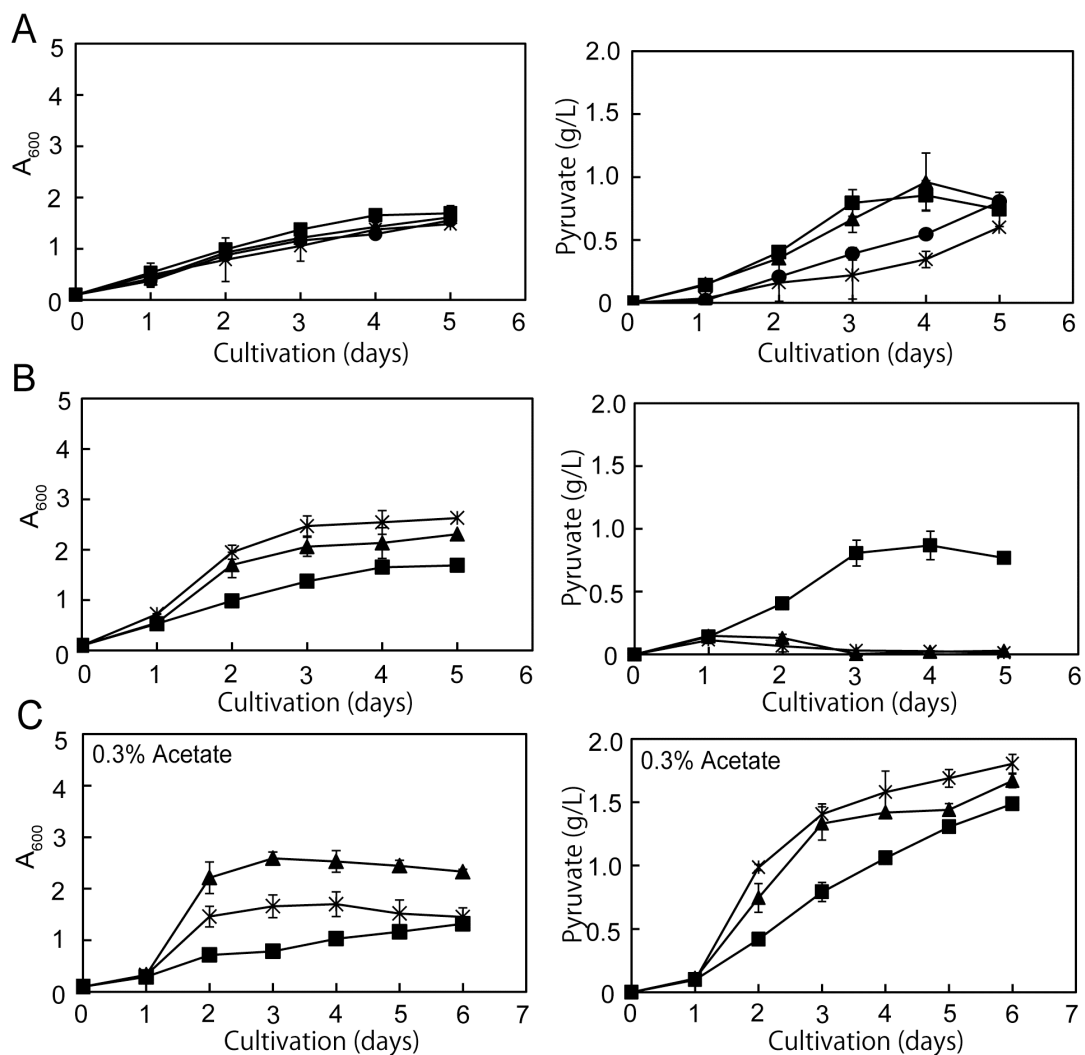
239

240 **Table 1.** *S. cerevisiae* strains used in this study

Strains	Descriptions	Sources
<i>S. cerevisiae</i>		
BY4742	MAT α <i>his3Δ1 his3Δ11leuΔ0 lys2Δ0 ura3Δ0</i>	Euroscarf
MK4416 ^a	BY4742 <i>cyc8</i> (Δ 1139-1164/ Q380ASCKTGRKX)	(14)
MK5286	MK4416 <i>ade2Δ0</i>	This study
MK5316	MK4416 <i>ade2Δ0 trp1Δ63</i>	This study
MK5327	MK4416 <i>ade2Δ0 trp1Δ63 pdc6Δ0</i>	This study
MK5336	MK4416 <i>ade2Δ0 trp1Δ63 pdc6Δ0 pdc1Δ0</i>	This study
MK5376	MK4416 <i>ade2Δ0 trp1Δ63 pdc6Δ0 pdc1Δ0</i>	This study
	<i>pdc5Δ::URA3</i>	

241 ^a MK4416 strain spontaneously lacks the central small region (1139-1164 nt) of
 242 *CYC8* (total, 2,901 nt) resulting in a nonsense mutation in which a stop codon was
 243 created after a short new peptide (³⁸⁰ASCKTGRK³⁸⁷)⁶. MK4416 strain acquired
 244 the ability to assimilate mannitol.

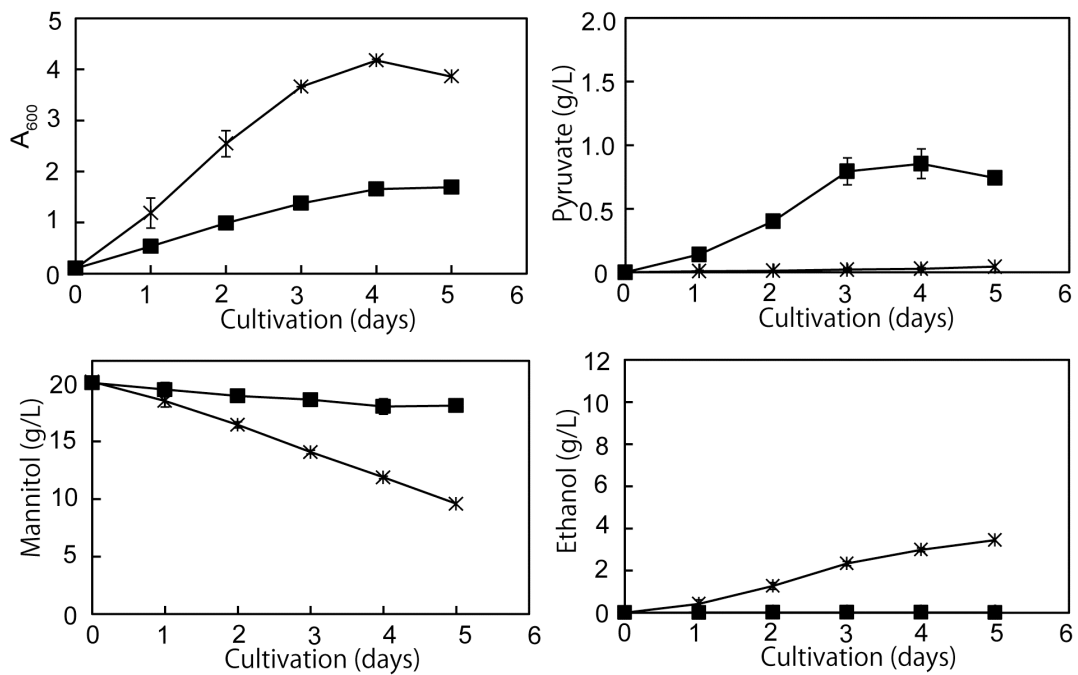
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245

246 **Fig. 1.** Production of pyruvate from mannitol by the mannitol-assimilating
 247 Pdc-negative MK5376 strain. Growth (left) and pyruvate concentration (right) for
 248 the MK5376 strain (A) precultured in several media, (B) cultured at several shaking
 249 speeds in SM medium, and (C) cultured in SM medium plus 0.3% w/v acetate.
 250 Pyruvate production was conducted as described in the Materials and Methods,
 251 except that (A) the MK5376 strain was precultured on SGE (0.3% ethanol) (closed
 252 circles), SGE (0.15%EtOH) (asterisks), SG (closed triangles), and SM (closed
 253 squares) solid media, further precultured in the same liquid media, and statically
 254 cultured at 0 rpm at 30°C; (B) cultivation was conducted at 0 (closed squares), 95

255 (asterisks), and 145 (closed triangles) spm at 30°C; and (C) cultivation was
256 conducted as in (B), but in the presence of 0.3% w/v acetate. (A-C) Averages and
257 maximum and minimum values are presented (n=2). (A) For precultivation in SM
258 medium, averages \pm standard deviations (SD) are presented (n=6).
259
260



260

261 **Fig. 2.** Comparison of growth profiles between the Pdc-negative MK5376 strain
262 and the Pdc-positive parental MK5316 strain. Pyruvate production was conducted
263 as described in the Materials and Methods. Averages and maximum and minimum
264 values (n=2) are presented, except that the growth and pyruvate concentration for
265 the MK5376 strain are averages \pm SD (n=6), and concentrations of ethanol and
266 mannitol for the MK5376 strain are averages \pm SD (n=3).

267

267 **Supplementary information for**

268

269 **Production of pyruvate from mannitol by mannitol-assimilating pyruvate**

270 **decarboxylase-negative *Saccharomyces cerevisiae***

271

272 **Shiori Yoshida, Hideki Tanaka, Makoto Hirayama, Kousaku Murata, Shigeyuki**

273 **Kawai**

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275

276

276 **Supplementary Methods**

277 Strains

278 Detailed information for plasmids and primers used in this study is provided in
279 Tables S1 and S2, respectively. Disruption of *ADE2*, *TRP1*, *PDC6*, and *PDC1* in
280 strain MK4416 was performed as previously described ¹. Briefly, the upstream
281 region (5'-1,993 bp to 5'-33 bp; ~2 kbp) and the downstream region (3'-69 bp to
282 3'-1,959 bp; ~2 kbp) of *ADE2* were PCR amplified from genomic DNA of BY4742
283 using primers 1 and 4, for the upstream region and primers 2 and 3 for the
284 downstream region. Using the amplified fragments as templates, a single DNA
285 fragment (4 kbp, the upstream region followed by the downstream region) was PCR
286 amplified using primers 1 and 2 and inserted into the EcoRI site of YIplac211 using
287 the In-fusion kit (Clontech), yielding pMK5265. After linearization of pMK5265
288 by EcoRI digestion, strain MK4416 was transformed with linearized pMK5265.
289 Transformants were selected on SC-U solid medium, purified again on the same
290 medium, and the Ade⁻ derivative was selected on SC+FOA solid medium, yielding
291 strain MK5286 (MK4416 *ade2Δ0*). Deletion of *ADE2* was confirmed by genomic
292 PCR using primers 1 and 2.

293 *TRP1*, *PDC6*, and *PDC1* were deleted using the same method. *TRP1* of MK5286
294 was deleted using pMK5300, and the Trp⁻ derivative was named MK5316
295 (MK5826 *trp1Δ63*). pMK5308 and pMK5329 were constructed as described above
296 for pMK5265 and linearized with EcoRI. Strain MK5316 was transformed with
297 linearized pMK5308, and transformants were selected on SC-U solid medium and
298 then MK5327 strain (MK5316 *pdcc6Δ0*) was selected. Deletion of *PDC6* was
299 confirmed with genomic PCR using primers 6 and 7 and by sequencing of the

300 resultant PCR products using primers 10 and 11. Similarly, strain MK5336
301 (MK5327 *pdclΔ0*) was obtained after transformation of strain MK5327 with
302 linearized pMK5329. Deletion of *PDC1* was confirmed by genomic PCR using
303 primers 12 and 13, and also by sequencing the resultant PCR products using primer
304 16.

305 Deletion of *PDC5* was conducted as described ². Briefly, the upstream region of
306 *PDC5* (5'-999 bp to 5'-4 bp; 1 kbp) and the *URA3* locus (1,037 bp) were PCR
307 amplified from genomic DNA of BY4742 and pRS416, respectively, using primers
308 24 and 25 for the upstream region and primers 26 and 27, for *URA3*. Using the
309 resultant DNA fragments as templates, a DNA fragment (*PDC5::URA3*; 2,110 bp)
310 was amplified using primers 24 and 27 and introduced into strain MK5336.
311 Transformants were selected on SGE-U (0.15% EtOH) solid medium, yielding
312 Pdc-negative strain MK5376 (MK5336 *pdcsΔ0*). Deletion of *PDC5* was confirmed
313 by genomic PCR using primers 24 and 27, and also by sequencing the resultant
314 PCR products using primers 5 and 28.

315

316 **Supplementary Results**

317 Pdc activity of cell extracts of parental MK5316 and Pdc-negative MK5376

318 Pdc activity of cell extracts of parental MK5316 was linear with respect to the
319 amount of protein in the reaction mixture (5.8–23.3 μg; Table S3). Based on these
320 data, specific activity of Pdc of MK5316 was calculated as 2.04 U/mg, slightly
321 lower than the reported activity (3.1 U/mg) in *S. cerevisiae* strain T2-3D ³. In
322 contrast to MK5316, cell extracts of Pdc-negative MK5376 exhibited lower activity
323 and no linearity. Even when 115.2 μg protein was present in the reaction mixture,

the specific activity was calculated as 0.01 U/mg. Thus, we concluded that the specific activity of MK5376 was undetectable.

Table S1. Plasmids used in this study

Plasmid	Descriptions	Sources
YIplac211	Amp ^r , <i>URA3</i>	Gietz <i>et al.</i> ⁵
pRS416	Amp ^r , <i>URA3</i> , <i>CEN</i>	Christianson <i>et al.</i> ⁶
pMK5265	The DNA fragment PCR-amplified using primers 1, 2, 3, and 4 was inserted into the EcoRI site of YIplac211 using the In-fusion kit. Only one EcoRI site is present 556 bp downstream of <i>ADE2</i> .	This study
pMK5300	Purchased from ATCC as ATCC77148 (YRp14/ <i>trp1Δ63</i>).	ATCC
pMK5308	The DNA fragment PCR-amplified using primers 6, 7, 8, and 9 was inserted into the EcoRI site of YIplac211 using the In-fusion kit. Only one EcoRI site is present 1,132 bp upstream of <i>PDC6</i> .	This study
pMK5329	The DNA fragment PCR-amplified using primers 12, 13, 14, and 15 was inserted into the EcoRI site of YIplac211 using the In-fusion kit. Only one EcoRI site is present 406 bp downstream of <i>PDC1</i> .	This study

330 **Table S2.** Primers used in this study

Nos	Primers	Sequences	Descriptions
1	YIpE15bF_ ADE2-1992 F	taccgagctcgaattTACG ATGTGGATGAGGG AG	For amplification of the upstream region of <i>ADE2</i> (5'-1,993 bp to 5'-33 bp) ^a .
2	YIpE15bR_ ADE2+1959 R	gacggccagtgaattGTG ACATCTAGACGCT CACAAG	For amplification of the downstream region of <i>ADE2</i> (3'-69 bp to 3'-1,959 bp) ^a .
3	ADE2-33F_ +69F	<u>GTACATCCTACTA</u> <u>TAACAATCAAGGT</u> TATGATTACATCA AATGTG	For amplification of the downstream region of <i>ADE2</i> , defined above. Sequence complementary to primer 4 is underlined.
4	ADE2-33R	CTTGATTGTTATA GTAGGATGTAC	For amplification of the upstream region of <i>ADE2</i> , defined above.
5	URA3_3_25 R	GTCGAAAGCTACA TATAAGGAAC	For sequencing.
6	YIpE15bF_ PDC6-1996F	taccgagctcgaattGCAG TGTCTGGTGTACC AC	For amplification of the upstream region of <i>PDC6</i> (5'-1,996 b to 5'-86 b) ^a .
7	YIpE15bR_ PDC6+1970 R	gacggccagtgaattACTT GAATTGTTCTTC AC	For amplification of the downstream region of <i>PDC6</i> (3'-112 b to 3'-1,970 b) ^a .

Table S2. *Continued.*

8	PDC6-86F	<u>GGCTGTTTGAAG</u>	For amplification of the downstream
	—	<u>CCATTCTATCCTA</u>	region of <i>PDC6</i> , defined above.
	+112F	AATACTACGTTA	Sequence complementary to primer 9
		TCGCCG	is underlined.
9	PDC6-86R	GATAGAATGGCT	For amplification of the upstream
		TCAAACAGCC	region of <i>PDC6</i> , defined above.
10	PDC6-308	GCCCACAACTTA	For sequencing.
	F	TCAAGTG	
11	PDC6+427	GCCAAAGAGATG	For sequencing.
	R	AGCCAAAG	
12	YIpE15bF_	taccgagctcgaattGTA	For amplification of the upstream
	PDC1-1932	TTGCAAGTGGTA	region of <i>PDC1</i> (5'-1,932 bp to 5'-27
	F	GTAC	bp) ^a .
13	YIpE15bR	gacggccagtgaattGAA	For amplification of the downstream
	—	CAGTTGTAGTAG	region of <i>PDC1</i> (3'-79 bp to 3'-1,998
	PDC1+199	CAC	bp) ^a .
	8R		
14	PDC1-27F	<u>CTACTCATAACC</u>	For amplification of the downstream
	—	<u>TCACGCCTGCAC</u>	region of <i>PDC1</i> (3'-79 bp to 3'-1,998
	+79F	TGTCACCTTACCAT	bp). Sequence complementary to
		G	primer 15 is underlined.
15	PDC1-27R	GCGTGAGGTTAT	For amplification of the upstream

GAGTAG region of *PDC1* (5'-1,932 bp to 5'-27 bp).

Table S2. *Continued.*

16	PDC1-209	CAGCTTATGGTG	For sequencing.
	F	ATGGCACA	
24	PDC5-999	GCCACGCTGATA	For amplification of the upstream
		GATATCCCG	region of <i>PDC5</i> (5'-999 bp to 5'-4 bp).
25	PDC5-4c	GTTCTTCTTGTTA	For amplification of the upstream
		TTGTATTGTG	region of <i>PDC5</i> above
26	PDC5URA	<u>CACAATACAATA</u>	For amplification of the <i>URA3</i> locus
	3-2	<u>ACAAGAAGAACTt</u>	(1,037 bp) from pRS416. Sequence
		tttacttttgaaggttatagat	complementary to primer 25 is
		gtttaggtaaataattGAT	underlined. The short downstream
		TCGGTAATCTCC	sequence of <i>PDC5</i> (3'-41 b to 3'-80 b)
		GA	is in lower case.
27	PDC5-40U	aatacacaacgttgaatcat	For amplification of the <i>URA3</i> locus
	c	gagttttatgttaattagcGG	from pRS416, defined above.
		GTAATAACTGAT	
		ATAATT	
28	PDC5_+36	CACAAACGTTGA	For sequencing.
	R	ATCATGAG	

331 ^a Sequence around *EcoRI* of YIplac211 is in lowercase.

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335 **Table S3.** Pdc activities of strains MK5316 and MK5376

Strains	Amounts of proteins	Activity
	in the reaction mixture (μg)	($\Delta m A_{340}/\text{min}$)
MK5316	5.8	178.4
	11.6	271.9
	23.3	503.0
MK5376	11.6	5.0
	115.2	11.9

336 Activity of MK5316 was assayed within 3 min, whereas activity of MK5376 was

337 assayed after a 10 min reaction.

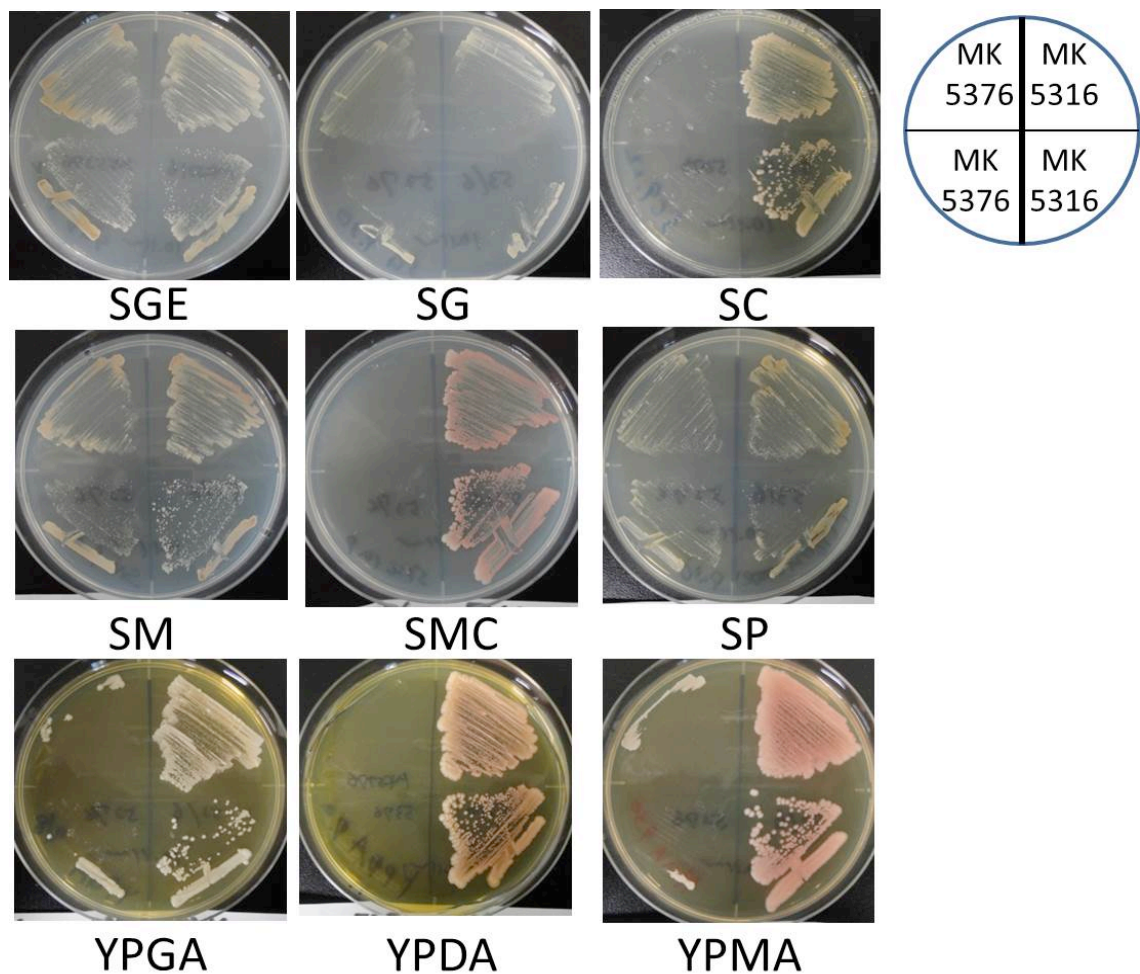
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Fig. S1. Growth phenotypes of the parental MK5316 strain and Pdc-negative MK5376 strain.

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MK5316 and MK5376 strains pre-grown on SGE solid medium were streaked with the indicated

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solid media and incubated at 30°C for 5 days. SGE, SG, SC, SM, SMC, and SP consisted of 0.67%

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w/v yeast nitrogen base w/o amino acids (BD), complete amino acids/nucleosides (Clontech), 2%

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w/v agar, and carbon sources: 2% v/v glycerol plus 0.15% v/v ethanol (SGE), 2% v/v glycerol (SG),

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2% w/v glucose (SC), 2% w/v mannitol (SM), 2% w/v mannitol plus 0.3% w/v glucose (SMC), and

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2% w/v sodium pyruvate. YPGA, YPDA, and YPMA solid media contained 2% w/v yeast extract,

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2% w/v tryptone, 147 mg/L adenine, 2% w/v agar (pH 5.6), and carbon sources: 3% v/v glycerol

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(YPGA), 2% w/v glucose (YPDA), and 2% w/v mannitol (YPMA). Representative results are

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shown.

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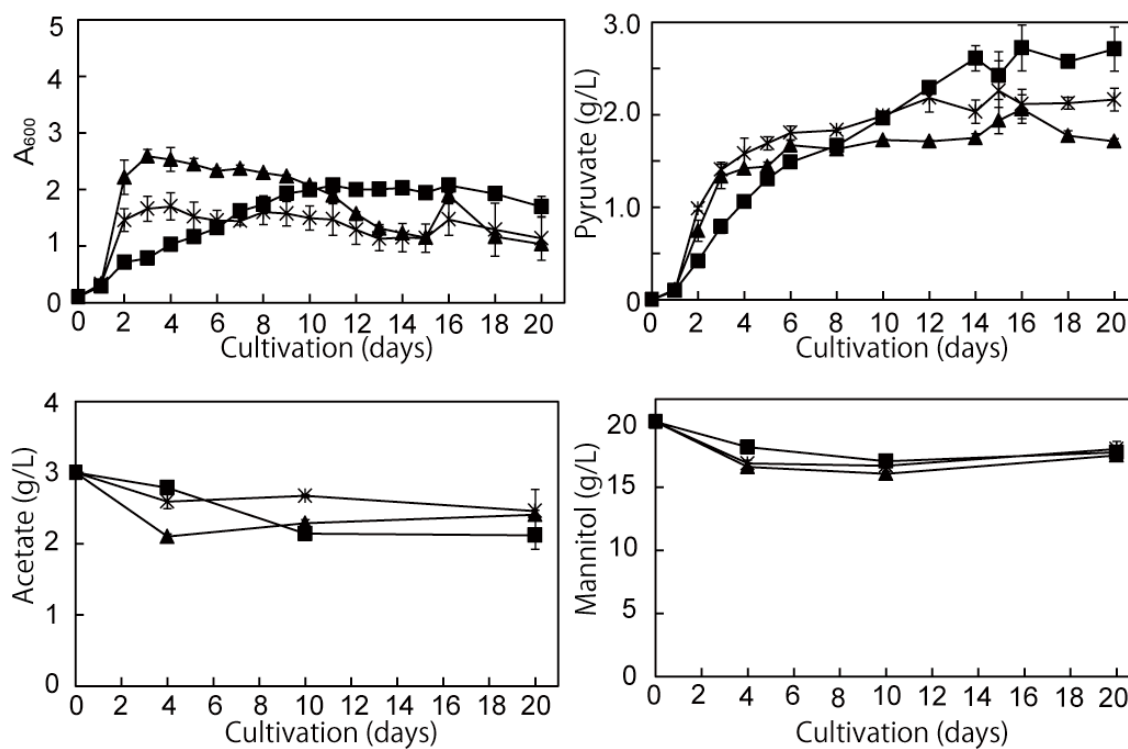


Fig. S2. Production of pyruvate from 2% w/v mannitol plus 0.3% w/v acetate. Production was conducted as in Fig. 1C and continued for longer periods, as indicated. Production of ethanol was not detected.

361 **Supplementary References**

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